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# Oxidation of Thiodiglycol (2,2'-Thiobis-ethanol) by Alcohol Dehydrogenase: Comparison of Human Isoenzymes

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**ABSTRACT:** Sulfur mustard is a chemical warfare agent that causes blistering of the skin and damages the eyes and airway after environmental exposure. We have previously reported that thiodiglycol (TDG, 2,2'-bis-thiodiethanol), the hydrolysis product of sulfur mustard, is oxidized by alcohol dehydrogenase (ADH) purified from horse liver or present in mouse liver and human skin cytosol. Humans express four functional classes of ADH composed of several different isozymes, which vary in their tissue distribution, some occurring in skin. To help us evaluate the potential contribution of the various human isozymes toward toxicity in skin and in other tissues, we have compared the catalytic activity of purified human class I  $\alpha\alpha$ -,  $\beta_1\beta_1$ -,  $\beta_2\beta_2$ -, and  $\gamma_1\gamma_1$ -ADH, class II  $\pi$ -ADH, class III  $\chi$ -ADH, and class IV  $\sigma$ -ADH with respect to TDG oxidation and their relative sensitivities to inhibition by pyrazole. Specific activities toward TDG were 123, 79, 347, 647, and 12 nmol/min/mg for the class I  $\alpha\alpha$ -,  $\beta_1\beta_1$ -,  $\beta_2\beta_2$ -, and  $\gamma_1\gamma_1$ -ADH and class II  $\pi$ -ADH, respectively. TDG was not a substrate for class III  $\chi$ -ADH. The specific activity of class IV  $\sigma$ -ADH was estimated at about 1630 nmol/min/mg. 1 mM pyrazole, a potent inhibitor of class I ADH, inhibited the class I  $\alpha\alpha$ -,  $\beta_1\beta_1$ -,  $\beta_2\beta_2$ -, and  $\gamma_1\gamma_1$  ADH and class IV  $\sigma$ -ADH by 83, 100, 56, 90, and 73%, respectively. The class I  $\alpha\alpha$ - and  $\beta_1\beta_1$ -ADH oxidized TDG with  $k_{cat}/K_m$  value of  $7-8 \text{ mM}^{-1} \text{ min}^{-1}$ ,  $\beta_2\beta_2$ -ADH with a value  $19 \text{ mM}^{-1} \text{ min}^{-1}$  and class I  $\gamma_1\gamma_1$ -ADH with a value of  $176 \text{ mM}^{-1} \text{ min}^{-1}$ . The  $k_{cat}/K_m$  value for class IV  $\sigma$ -ADH was estimated at  $4 \text{ mM}^{-1} \text{ min}^{-1}$ . The activities of class IV  $\sigma$ -ADH and class I  $\gamma_1\gamma_1$ -ADH are of significant interest because of their prevalence in

eyes, lungs, stomach, and skin, all target organs of sulfur mustard toxicity. © 2000 John Wiley & Sons, Inc. J Biochem Toxicol 14:244-251, 2000

**KEY WORDS:** Human ADH; Sulfur Mustard; Thiodiglycol; Chemical Warfare Agents

## INTRODUCTION

Sulfur mustard is a chemical warfare agent that exerts its effects primarily on the skin, eyes, and airways of those exposed. High doses by the cutaneous route and parenteral administration result in systemic effects on the digestive system and on hemopoietic tissue. Convulsions and cardiac irregularities have been reported, but only after extremely high doses probably attainable only under laboratory conditions [1]. Recently the Iraqi Army employed sulfur mustard against Iranian forces during the Iran-Iraq War with the production of an estimated 45,000 casualties [2].

In an aqueous environment, be it tissue or environmental moisture, sulfur mustard hydrolyzes to form 2,2'-thiobis-ethanol (thiodiglycol, TDG), which is relatively nontoxic. Recent research indicated inhibition of protein (serine/threonine) phosphatases in tissue cytosol by sulfur mustard *in vitro* [3]. These enzymes have been implicated in the mechanism of vesication because they are inhibited by the natural vesicant cantharidin [4] and by the chemical warfare agent Lewisite, also a vesicant [5]. Interestingly, in the work with sulfur mustard, inhibitory activity correlated with the concentration of TDG rather than with that of mustard itself [3]. Unsuccessful attempts to replicate the inhibitory effect of TDG on purified preparations of protein phosphatases 1 and 2A (A. Brimfield,

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unpublished results, 1998) rather than cytosol have prompted the study of the metabolism of TDG by cytosolic enzymes. Subsequent observations have shown TDG to be a substrate for horse liver alcohol dehydrogenase (ADH) and ADH in human skin and mouse liver cytosol [6]. TDG oxidation by the horse liver enzyme and the cytosols were sensitive to inhibition by 4-methyl pyrazole.

Mammalian zinc-containing ADHs constitute an enzyme family of multiple isoforms. Humans produce more than 20 ADH isozymes composed of as many as nine different subunits [7]. They have been assigned to four functional classes based on their genetics, electrophoretic mobilities, and responses to specific inhibitors. The overall residue identity shows 60% homology among the classes. The human ADH classes also differ in their tissue distribution, although individual isozymes are identical regardless of their location [8–10]. Extensive work has been done to characterize the catalytic properties, expression, and localization of ADH in mammals [11–14]. Recently, Cheung et al. [15] characterized various ADH content and distribution in human skin.

The class I  $\alpha\alpha$ -,  $\beta_1\beta_1$ -,  $\beta_2\beta_2$ -, and  $\gamma_1\gamma_1$ -ADHs [16,17] are effectively inhibited by pyrazole and its 4-alkylated derivatives. Homodimers and heterodimers of  $\alpha$ ,  $\beta$ , and  $\gamma$  subunits of human class I ADH are present in liver, kidney, skin, gastrointestinal tract, and to a lesser extent, lung [18]. Class II and class III ADH each consist of one isozyme,  $\pi$ -ADH and  $\chi$ -ADH, respectively, and are relatively insensitive to inhibition by pyrazole or its 4-alkylated derivatives [19,20]. All human ADHs catalyze the oxidation of ethanol; albeit at different levels of activity based on their  $K_m$  and  $V_{max}$  values. Of the vertebrate ADHs that have been identified, only class III ADH has been conserved in all organisms [21]. Class IV  $\sigma$ -ADH has exceptionally high  $K_m$  and  $k_{cat}$  values for ethanol and alcohols and aldehydes of physiological interest. Found predominately in the stomach, the  $\sigma$ -ADH is considered to have a significant role in retinol metabolism and first-pass ethanol metabolism [22]. Here we have compared the catalytic ability of purified  $\alpha\alpha$ -,  $\beta_1\beta_1$ -,  $\beta_2\beta_2$ -,  $\gamma_1\gamma_1$ -,  $\chi$ -,  $\pi$ -, and  $\sigma$  human ADH isozymes to oxidize TDG in an effort to further identify and characterize the sources of TDG transformation in skin and other tissues.

## MATERIALS AND METHODS

### Chemicals and Enzymes

Tris-maleate and  $\beta$ -nicotinamide adenine dinucleotide were obtained from Sigma Chemical Co. (St. Louis, MO). Thiodiglycol and pyrazole and 4-methyl

pyrazole were from Aldrich Chemical Co. (Milwaukee, WI). Horse liver alcohol dehydrogenase was obtained from Boehringer-Mannheim (Indianapolis, IN). The horse liver ADH used in the dose-response work was from Sigma Chemical Co. All other chemicals were of the highest grade commercially available. Purified human liver ADH isozymes were generously provided for these studies by Dr. Thomas Hurley, Department of Biochemistry and Molecular Biology, Indiana University School of Medicine.

### Enzyme Assays

Thiodiglycol oxidation by ADH was determined essentially according to Brimfield et al. [6] and modified for use in microtiter plates. All solutions were prewarmed to 30°C. A 200  $\mu$ L aliquot of 5 mM  $NAD^+$  in 20 mM tris-maleate buffer, pH 7.6, was placed in each experimental well of a 96-well microtiter plate, followed by 25  $\mu$ L of enzyme solution at the concentrations indicated in the figure and table legends in 20 mM tris-maleate buffer, pH 7.6. A baseline was determined after a 2 minutes preincubation period in a THERMOMax MAXline Molecular Dynamics Microplate Reader running SOFTmax software and equipped for constant temperature. At the end of the preincubation, 25  $\mu$ L of the appropriate concentration of substrate in 0.85% saline was added. The reaction was followed by measuring the change in absorbance at 340 nm for 5 minutes with mixing before each reading. Under the described conditions the ADH-catalyzed reactions were linear for at least 15 minutes and first order with respect to enzyme concentration. In reactions containing pyrazole, the volume of the  $NAD^+$  solution added was reduced by 10  $\mu$ L, and 10  $\mu$ L of the appropriate concentration of inhibitor in tris-maleate buffer was added before the 2 minute incubation. Ethanol oxidation was measured by the method of Bonnichsen et al. [23], which is based on spectrophotometric measurement of NADH formation from  $NAD^+$  in the presence of ethanol.

### Generation of Dose-Response Curves for ADH Inhibition by 4-Methyl Pyrazole

Dose-response curves for the inhibition of TDG oxidation by the purified horse liver enzyme and in mouse liver and human skin cytosols were carried out to compare the relative levels of inhibition obtainable. Mouse liver cytosol was made from male CD-1 mice (Jackson Laboratories, Bar Harbor, ME). Mice were housed in AAALAC-approved facilities with feed and water provided ad libitum. Human skin samples (OSU Tissue Services, Columbus, OH) were collected in conjunction with surgical correction of macromastia.

**TABLE 1.** Specific Activities of ADH-Catalyzed Oxidation of Thiodiglycol and Inhibition by Pyrazole (1mM)<sup>a</sup>

Class	ADH isozyme	Specific Activity (nmol/min/mg)	% Pyrazole Inhibition	Alcohol Oxidation <sup>b</sup> (μmol/min/mg)
I	αα	123 (146 ± 5) <sup>c</sup>	83	0.58
	β <sub>1</sub> β <sub>1</sub>	79 (86 ± 2)	100	0.11
	β <sub>2</sub> β <sub>2</sub>	347 (505 ± 20)	56	5.64
	γγ	647 (617 ± 12)	90	2.06
II	π	12 (ND)	0	0.11
III	χ	No activity	—	2.70 <sup>d</sup>
IV	σ	1630 (2850 ± 470)	73	15.40

<sup>a</sup>Activities are means of triplicate measurements. Specific activities of TDG were determined at: 45 mM TDG with 2.8 μg/mL αα, 45 mM TDG with 6.83 μg/mL β<sub>1</sub>β<sub>1</sub>, 200 mM TDG with 6.9 μg/mL β<sub>2</sub>β<sub>2</sub>, 33.4 mM TDG with 1.6 μg/mL γγ, 33.4 mM TDG with 4.4 μg/mL π, and 400 mM TDG with 0.21 μg/mL σ-ADH.

<sup>b</sup>All alcohol oxidase activity measurements were in 100 mM Glycine, pH 10; except β<sub>2</sub>β<sub>2</sub>, which was in 100 mM sodium phosphate, pH 8.5.

<sup>c</sup>Values in parentheses are  $V_{max}$  values ± S.E. calculated from hyperbolic curve fits of the data with the software *Enzyme Kinetics* (version 1.2) by Cleland [24]. The good agreement of the specific activities with  $V_{max}$  values indicate that reactions were conducted under saturating substrate conditions.

<sup>d</sup>Alcohol oxidase activity measurements for χ-ADH were performed using cinnamyl alcohol.

Cytosol (105,000 × *g* supernatant) was prepared by differential centrifugation according to standard techniques.

## Data Analysis

Kinetic constants for TDG were determined by monitoring the production of NADH at 340 nm ( $\epsilon = 6.32 \text{ mM}^{-1} \text{ cm}^{-1}$ ).  $K_m$  and  $V_{max}$  values for TDG were calculated by fitting all kinetic data to the Michaelis-Menten equation [ $v = V_{max} S / (K_m + S)$ ], where  $S$  is the concentration of TDG, by using the statistical software *Enzyme Kinetics* (version 1.2) by Cleland [24]. These kinetic constants were confirmed from a fit of the kinetic data to the Lineweaver-Burk equation [ $1/v = K_m / (V_{max} S) + 1/V_{max}$ ] and from Hanes-Woolf plots [ $S \text{ vs. } v/S$ ]. The value of  $k_{cat}$  ( $\text{min}^{-1}$ ) was obtained by dividing  $V_{max}$  by the concentration of active sites assuming a subunit MW of 40,000.  $K_i$  values for inhibition by pyrazole were calculated from Dixon plots [25].

## RESULTS

### Oxidation of TDG by Purified Human ADH Isozymes

The specific activities (Table 1) of each of the purified ADH isozymes were determined at saturating concentrations of TDG. Of the ADH isozymes, class IV σ-ADH exhibited the highest specific activity, at 1630 nmol/min/mg in the presence of 400 mM TDG. The specific activities of the class I ADH were, respectively 123, 79, 347, and 647 nmol/min/mg for the αα, β<sub>1</sub>β<sub>1</sub>,

β<sub>2</sub>β<sub>2</sub> and γγ isozymes. The values in parentheses are  $V_{max}$  values calculated from hyperbolic curve fits of the data with the software *Enzyme Kinetics* (version 1.2) by Cleland [24]. The good agreement between the specific activities reported and the  $V_{max}$  values indicate that the experiments were indeed conducted under conditions of saturating substrate concentration. Class II π-ADH exhibited the lowest activity; 12 nmol/min/mg protein. Class III χ-ADH was inactive toward this substrate even when followed for 1 hour with 100 mM TDG and elevated levels of enzyme. The absence of TDG oxidation seen with class III χ-ADH was apparently not due to lack of enzymic integrity as evinced by the high activity displayed with cinnamyl alcohol, a preferred substrate for this isozyme [20]. The relative orders of activity toward TDG catalyzed by the various human ADH were essentially the same as that for ethanol oxidation (Table 1), with the class I γγ-ADH being 4- to 5-fold greater than αα-ADH and β<sub>1</sub>β<sub>1</sub>-ADH having notably lower activity. The specific activity for β<sub>2</sub>β<sub>2</sub>-ADH was about half that of γγ-ADH, whereas β<sub>2</sub>β<sub>2</sub>-ADH oxidized ethanol at a rate twice that of γγ-ADH. The specific activity of class IV σ-ADH was approximately three times greater with respect to TDG oxidation and seven times greater with respect to ethanol oxidation than that of class I γγ-ADH.

### Kinetic Constants for ADH-Catalyzed Oxidation of TDG

Based on the specific activity measurements of Table 1, only the class I and class IV ADH were subjected to more detailed kinetic analysis. The class I isozymes appeared to exhibit classical Michaelis-Menten kinetics (Figure 1). The  $K_m$  values of TDG derived from Lineweaver-Burk plots for the class I isozymes αα, β<sub>1</sub>β<sub>1</sub>, and γγ ranged from 1.2 to 8.6 mM (Table 2). These were in good agreement with values obtained from hyperbolic fits of the data by use of Cleland's statistical program [24]. The highest  $K_m$  value for TDG oxidation among the class I isozymes was for β<sub>2</sub>β<sub>2</sub>-ADH (47 mM).

Caution needs to be exercised when interpreting the kinetic data of σ-ADH. At high concentrations, ethanol displays substrate inhibition of other ADH [26]. The σ isozyme did not appear to display classical Michaelis-Menten kinetics; it showed a linear increase in activity up to approximately 200 mM approaching its highest velocity at TDG concentrations in the 300–400 mM range (Figure 2). Above 600 mM the reaction was noticeably inhibited, for example, at 800 mM the rate fell noticeably when compared with that at 400 mM. Therefore, the maximum activity in the  $v \text{ vs. } S$  plot for σ-ADH may not be a true reflection of saturation kinetics. Perturbations caused by the very high concen-

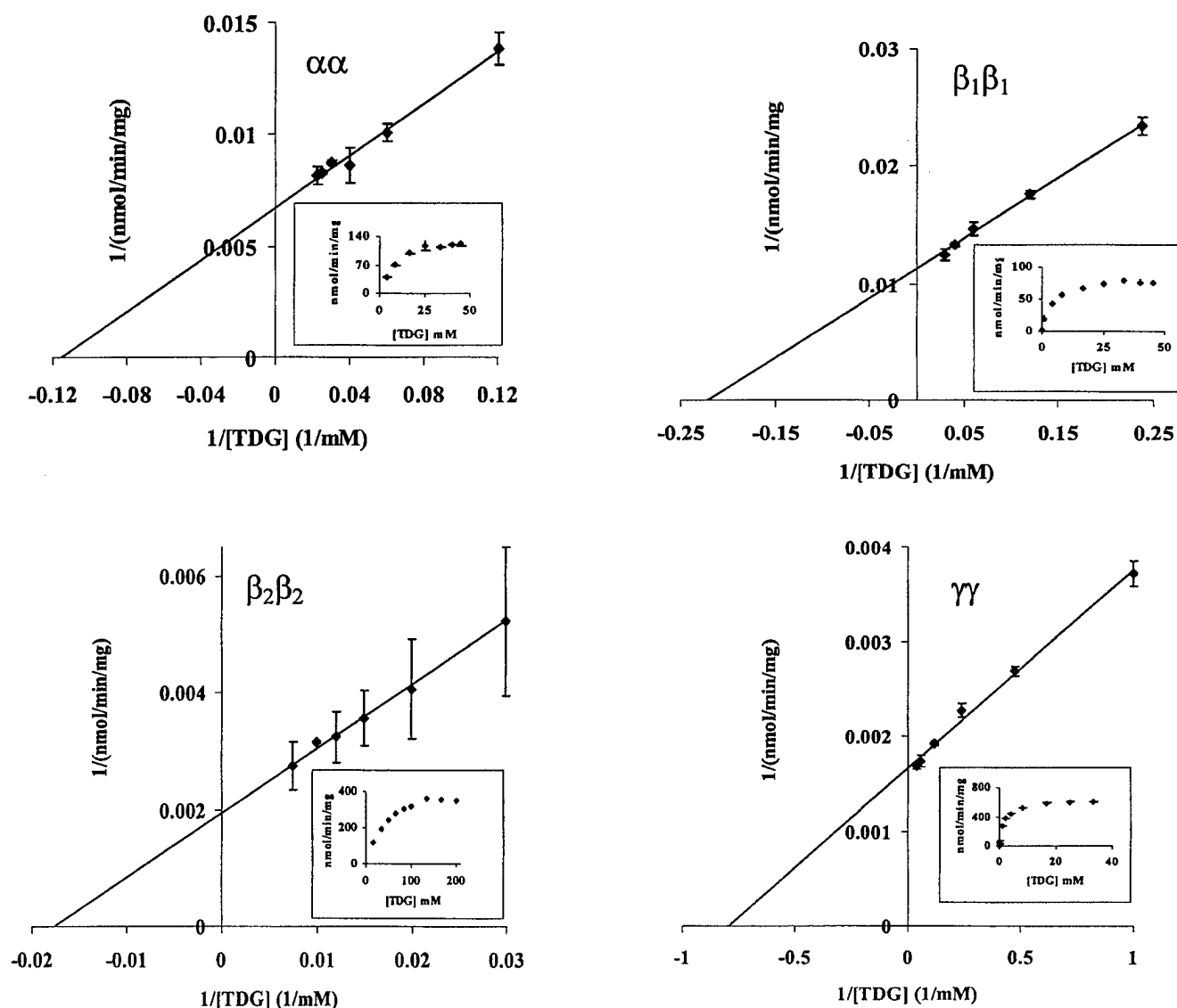


FIGURE 1. Lineweaver-Burk plots of oxidation of thiodiglycol by human ADH. The experiments were conducted as described in Materials and Methods under the following conditions. Upper left, 4.2–45 mM TDG; 2.8  $\mu\text{g/mL}$   $\alpha\alpha$  ADH; upper right, 8.3  $\mu\text{M}$ –45 mM TDG; 6.8  $\mu\text{g/mL}$   $\beta_1\beta_1$  ADH; lower left, 16.7 mM–200 mM TDG; 6.9  $\mu\text{g/mL}$   $\beta_2\beta_2$  ADH; lower right, 4.2 mM–45 mM TDG; 1.55  $\mu\text{g/mL}$   $\gamma\gamma$  ADH. All reactions contained 5 mM  $\text{NAD}^+$  in 20 mM tris-maleate buffer, pH 7.6. (Insert) Michaelis-Menten plot of  $v$  vs.  $S$  for the oxidation of TDG by human ADH. Each data point represents the mean  $\pm$  SD of at least three trials.

trations of substrate may also be a tenable explanation. If the latter is true, then calculation of a  $K_m$  and hence catalytic efficiency based on typical Michaelis-Menten approximations are not applicable. We suggest that  $\sigma$ -ADH may be highly efficient and not saturable by TDG even at high mM concentrations. That this hypothesis has credence is suggested by the relative efficiencies ( $k_{\text{cat}}/K_m$ ) observed with longer chain (4–8 C) aliphatic alcohols and  $\sigma$ -ADH, which are more than two orders of magnitude greater than that obtained with ethanol [18]. In this regard, TDG most closely resembles a 5C aliphatic alcohol in its structural motif. Nevertheless, when the data for the  $v$  vs.  $S$  plot for TDG oxidation

by  $\sigma$ -ADH shown in Figure 2 were subjected to the statistical program of Cleland [24], a reasonable estimate of  $V_{\text{max}}$  was obtained (ca. 2850 nmol/min/mg protein;  $k_{\text{cat}} \approx 1500 \text{ min}^{-1}$ ). Class IV  $\sigma$ -ADH also exhibited the highest  $K_m$  with respect to TDG of all the isoforms studied ( $>250 \text{ mM}$ ), that is, two orders of magnitude higher than those of the class I ADH. This was consistent with the substantially higher  $K_m$  for ethanol oxidation by  $\sigma$ -ADH compared with the other human enzymes (Table 1).

Among the class I ADH, the  $\alpha\alpha$  and  $\beta_1\beta_1$  isoforms oxidized TDG with approximate  $k_{\text{cat}}/K_m$  values of 7–8  $\text{mM}^{-1} \text{ min}^{-1}$ ; the value for  $\beta_2\beta_2$  was 19  $\text{mM}^{-1} \text{ min}^{-1}$ ,

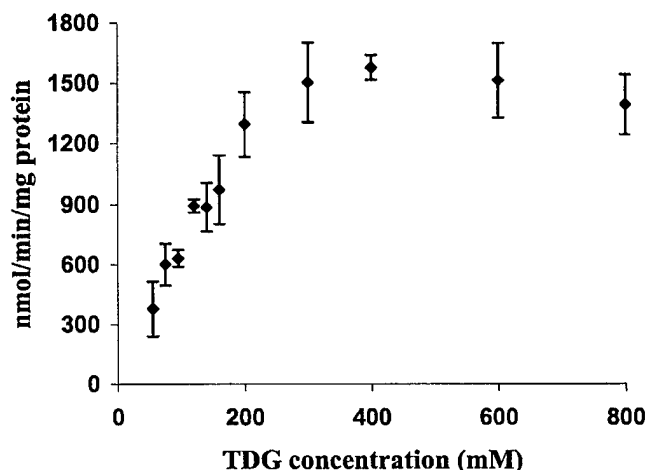
**TABLE 2.** Kinetic Parameters for the Oxidation of Thiodiglycol and Ethanol by ADH Isozymes<sup>a</sup>

Class	Isozyme	TDG			Ethanol		
		$K_m^b$	$k_{cat}$	$k_{cat}/K_m$	$K_m$	$k_{cat}$	$k_{cat}/K_m$
I	$\alpha\alpha$	8.6 (7.9 $\pm$ 1.2) <sup>c</sup>	63	7	4.2	54	13
	$\beta_1\beta_1$	3.5 (4.2 $\pm$ 0.4)	34	8	0.05	18	376
	$\beta_2\beta_2$	47 (37 $\pm$ 5)	505	19	1.2	696	580
	$\gamma_1\gamma_1$	1.2 (1.4 $\pm$ 0.1)	267	176	1.1	140	130
IV	$\sigma$	256 (293 $\pm$ 86)	1500	4	41	1000	24

<sup>a</sup>Units for kinetic constants:  $K_m$ , mM;  $k_{cat}$ , min<sup>-1</sup>;  $k_{cat}/K_m$ , mM<sup>-1</sup> min<sup>-1</sup>. Enzyme concentrations used were: 2.8, 6.8, 6.9, 1.6, and 0.2  $\mu$ g/mL, respectively, for  $\alpha\alpha$ -,  $\beta_1\beta_1$ -,  $\beta_2\beta_2$ -,  $\gamma_1\gamma_1$ -, and  $\sigma$ -ADH. Data for ethanol oxidation catalyzed by  $\alpha\alpha$ -,  $\gamma_1\gamma_1$ -, and  $\sigma$ -ADH were from Pares *et al.* [28], for  $\beta_1\beta_1$  from Bosron *et al.* [29], for  $\beta_2\beta_2$  from Kedishvili *et al.* [27].

<sup>b</sup> $K_m$  values obtained from Lineweaver-Burk plots (Figure 1).

<sup>c</sup>Values in parentheses are  $K_m \pm$  S.E. calculated from hyperbolic curve fits of the data with the software *Enzyme Kinetics* (version 1.2) by Cleland [24].  $k_{cat}/K_m$  values were calculated using the  $K_m$  obtained from the Cleland program.

**FIGURE 2.** Michaelis-Menten plot (*v* vs. *S*) for TDG oxidation by Class IV  $\sigma$ -ADH. The final reaction mixture consisted of 75–600 mM TDG; 0.21  $\mu$ g/mL  $\sigma$  ADH in a 200  $\mu$ L reaction volume. Data points are means of 3–5 determinations. The experiment was conducted as described in Materials and Methods.

and  $\gamma_1\gamma_1$  had a markedly higher value, 176 mM<sup>-1</sup> min<sup>-1</sup> that is, the highest efficiency for TDG oxidation of all the human ADH studied (Table 2). The  $k_{cat}/K_m$  for  $\sigma$ -ADH was about 4 mM<sup>-1</sup> min<sup>-1</sup>, albeit the same caveats described previously for this isozyme apply.

### Inhibition by Pyrazole of TDG Oxidation by Human ADH

Using the most active isozymes, we determined that 1 mM pyrazole, a potent inhibitor of ADH, inhibited the class I  $\alpha\alpha$ -,  $\beta_1\beta_1$ -,  $\beta_2\beta_2$ -, and  $\gamma_1\gamma_1$ -ADH, and class IV  $\sigma$ -ADH by 83, 100, 56, 90, and 73%, respectively (Table 1). To conserve purified human ADH the apparent  $K_i$  for pyrazole inhibition of TDG oxidation was

**TABLE 3.** Kinetic Constants for the Oxidation of Thiodiglycol by ADH Isozymes Determined from Hyperbolic Curve Fits

Class	Isozyme	$K_m$	$V_{max}$	S.E.
I	$\alpha\alpha$	7.9	146	1.2, 5.3
	$\beta_1\beta_1$	4.2	86	0.35, 1.7
	$\beta_2\beta_2$	54	842	5.2, 20
	$\gamma_1\gamma_1$	1.4	617	0.12, 12
IV	$\sigma$	293	2850	87, 467 +

**Apparent  $K_i$  Values for the Inhibition of TDG Oxidation by Pyrazole<sup>a</sup>**

Class	Isozyme	$K_i$ , Pyrazole
I	$\alpha\alpha$	82
	$\beta_2\beta_2$	430
	$\gamma_1\gamma_1$	3.2
IV	$\sigma$	878

<sup>a</sup> $K_i$  units are  $\mu$ M. The experiments were conducted as described in Materials and Methods, and  $K_i$  values were calculated from Dixon plots. Enzyme concentrations used were: 2.8  $\mu$ g/mL  $\alpha\alpha$ , 6.9  $\mu$ g/mL  $\beta_2\beta_2$ , 1.6  $\mu$ g/mL  $\gamma_1\gamma_1$ , and 0.21  $\mu$ g/mL  $\sigma$ -ADH.

determined for  $\alpha\alpha$ -,  $\beta_2\beta_2$ -,  $\gamma_1\gamma_1$ -, and  $\sigma$ -ADH, that is, the most active isozymes in TDG oxidation from Dixon plots (not shown). The apparent  $K_i$  values for class I  $\gamma_1\gamma_1$ -,  $\beta_2\beta_2$ -, and  $\alpha\alpha$ -ADH were, respectively 3.2, 430, and 82  $\mu$ M, and for the class IV  $\sigma$ -ADH, about 878  $\mu$ M (Table 3). Precise determination of  $K_i$  for pyrazole inhibition of  $\sigma$ -ADH was hampered by the extremely high concentrations of TDG required; thus, very high pyrazole concentrations were necessary to compete with substrate. Furthermore, the high activity of the  $\sigma$  isozyme may also lead to higher concentrations of NADH being formed from the oxidation of TDG, with subsequent competitive inhibition toward NAD<sup>+</sup> in the forward reaction. Under the described experimental conditions, the oxidation of TDG by  $\beta_1\beta_1$ -ADH was inhibited 100% by 0.4 mM pyrazole. Lower concentrations were not used, but a  $K_i$  value much lower than 0.4 mM is indicated. As expected, 1 mM pyrazole was without effect on the class II ADH, a known pyrazole-insensitive isozyme [19].

### Inhibition of ADH Activity in Cytosolic Preparations by 4-Methylpyrazole

Dose-response curves produced using cytosol from mouse liver or human skin (Figure 3) provided a system containing a mixture of ADH that more closely resembles the situation actually found in tissues. The enzyme from horse liver, known to be sensitive to inhibition by pyrazole and its 4-alkylated derivatives, served as a positive control. While the activity of the purified enzyme could be virtually abolished by 4-methylpyrazole (4MP), the ADH activity in the cytosolic preparations was incompletely inhibited. The activity in mouse liver reached an asymptote after elimination of about 65% of the total activity at a concentration near 100 mM 4MP. Human skin cytosol had

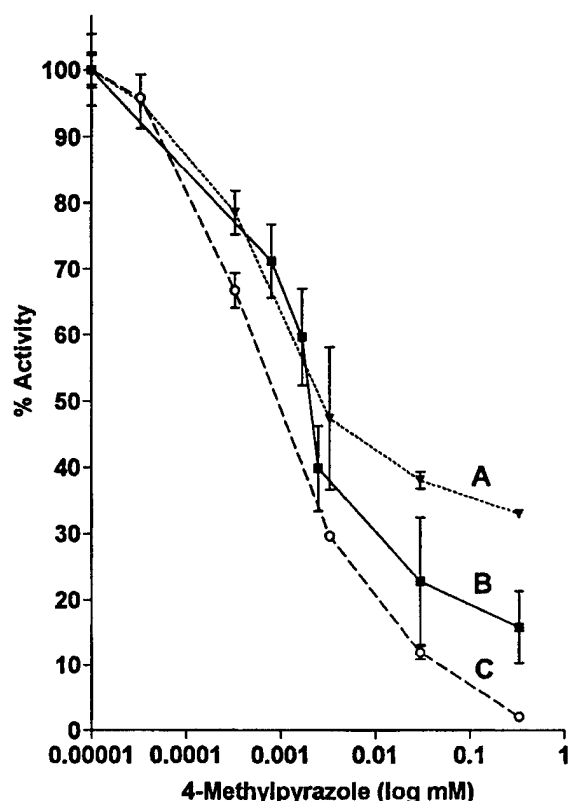


FIGURE 3. Inhibition of TDG dehydrogenase activity of (A) mouse liver cytosol, (B) human skin cytosol, and (C) purified horse liver ADH by varying concentrations of 4-methyl pyrazole. Conditions are described in Materials and Methods. Each data point represents the mean  $\pm$  S.D. for at least three trials.

a residual 4MP-insensitive activity below about the 85% inhibition level at the same inhibitor concentration. In each case, this indicates an increment of pyrazole-insensitive ADH and suggests activity from class II  $\pi$ - and/or class III  $\chi$ -ADH. Since TDG was not a substrate for the purified human class III isozyme (Table 1), it appears that the 4MP-insensitive activity reflects the action of the class II isozyme and its murine analog in the liver cytosol.

## DISCUSSION

We have compared the kinetics of TDG oxidation by several isozymes representative of classes I-IV of human ADH. This study is pursuant to a previous investigation [6] of the mechanism of vesication by sulfur mustard, which showed that an enzyme or enzymes of human skin cytosol catalyzed NAD<sup>+</sup>-dependent oxidative transformation of TDG, the hydrolysis product of sulfur mustard. In the same work it was demonstrated that TDG acted as a substrate for the purified enzyme from horse liver and for cytosol prepared from

mouse liver. Recently, Cheung et al. [15] identified ADH of classes I, II, and III in human skin samples by Western blot analysis and immunohistochemical staining using class-specific antisera. It was of interest therefore, to establish the pattern of specificity for TDG among the various classes of human ADH to better elucidate the possible sources of activity in the skin and other tissues. All of the purified human class I catalyzed TDG oxidation. The Cheung study [15] clearly showed the presence of class I ADH in skin. However, the limitations in the specificity of the antisera used did not permit differentiation of the various ADH isoenzymes within class I.

The highest efficiency for TDG oxidation by the various purified human ADH studied was observed with the class I  $\gamma_1\gamma_1$ -ADH. This arises from the fact that this isozyme had the lowest  $K_m$  and the highest  $k_{cat}$  among the class I enzymes (Table 2). Moreover, the high catalytic efficiency of  $\gamma_1\gamma_1$ -ADH coupled with its substantial presence in human skin [15] suggests a marked potential for TDG oxidation by the skin. Class III  $\chi$ -ADH did not catalyze TDG oxidation; thus, despite its presence in skin and other tissues, it seems unlikely that this isozyme has a role in the metabolism of TDG. Both the  $\alpha\alpha$  and  $\beta_2\beta_2$  isozymes showed moderate TDG oxidase activity; however, when compared with the  $\gamma_1\gamma_1$  isozyme, their catalytic efficiencies were more than an order of magnitude lower. In light of these results it appears that among the class I isozymes  $\gamma_1\gamma_1$ -ADH would likely account for the major portion of class I activity in the skin. Furthermore, the low  $K_i$  value (3  $\mu$ M) obtained for pyrazole inhibition with  $\gamma_1\gamma_1$ -ADH (Table 3) suggests a possible therapeutic role for pyrazole and 4-substituted pyrazoles in sulfur mustard toxicity if, indeed, ADH-dependent TDG metabolism is part of the toxic sequelae of sulfur mustard exposure.

Class II  $\pi$ -ADH catalyzed very low level TDG oxidation (12 nmol/min/mg) and is pyrazole-insensitive. Class II ADH is present in human epidermis [15] and may be responsible for residual activity remaining in skin cytosol after treatment with 4-methyl pyrazole. However the low specific activity catalyzed by class II ADH (Table 1) suggests only a minor role.

Because of the severe vesicating action of sulfur mustard, our focus to this point has been on the toxicity of sulfur mustard to skin. Environmental exposure to mustard also causes toxicity to the eyes, lungs, and airway. Class IV  $\sigma$ -ADH is the extrahepatic form of ADH found in epithelial cells, especially in the lining of the stomach and esophagus [27]. However,  $\sigma$ -ADH protein and mRNA have been detected in the eye and skin [8,28], both major target organs of sulfur mustard toxicity. In light of the very high specific activity of class IV  $\sigma$ -ADH toward oxidation of TDG (Table 1),

turnover of TDG could exceed that of the other human ADH isozymes even at very low TDG concentrations, that is, well below the estimated  $K_m$  for this isozyme (Table 2). This indicates the potential for a significant volume of TDG transformation by the eye, skin, or any other target organ of sulfur mustard toxicity that contains class IV ADH. Systemically, and after parenteral administration, effects are seen in the gastrointestinal system, hematopoietic tissues, and kidneys [1,2], the so-called radiomimetic effects. This organotropy has been attributed to the ability of sulfur mustard to act as an alkylating agent causing nucleic acid damage to cells in rapidly dividing tissues. Nevertheless, the tissue distribution of the various alcohol dehydrogenases gives us another framework in which to view the systemic effects of sulfur mustard.

Based on this work, the likelihood is that the major source of ADH-dependent TDG oxidation in human skin cytosol resides in one or more of the class I enzymes and in some tissues, such as skin, eye, gastrointestinal mucosa, as well as in the class IV enzyme. The exact role that the ADH-catalyzed oxidation of TDG plays in sulfur mustard toxicity has not been established. These studies may prove important in the interpretation of the interactions between sulfur mustard and metabolic systems of humans.

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